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Resistance to Broad-Spectrum Antibiotics in Aquatic Systems: Anthropogenic Activities Modulate the Dissemination of *bla*$_{CTX-M}$-Like Genes

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We compared the resistomes within polluted and unpolluted rivers, focusing on extended-spectrum beta-lactamase (ESBL) genes, in particular *bla*$_{CTX-M}$. Twelve rivers from a Portuguese hydrographic basin were sampled. Physicochemical and microbiological parameters of water quality were determined, and the results showed that 9 rivers were classified as unpolluted (UP) and that 3 were classified as polluted (P). Of the 225 cefotaxime-resistant strains isolated, 39 were identified as ESBL-producing strains, with 18 carrying a *bla*$_{CTX-M}$ gene (15 from P and 3 from UP rivers). Analysis of CTX-M nucleotide sequences showed that 17 isolates produced CTX-M from group 1 (CTX-M-1, -3, -15, and -32) and 1 CTX-M that belonged to group 9 (CTX-M-14). A genetic environment study revealed the presence of different genetic elements previously described for clinical strains. IS*Ecp1* was found in the upstream regions of all isolates examined. Culture-independent *bla*$_{CTX-M}$-like libraries were comprised of 16 CTX-M gene variants, with 14 types in the P library and 4 types in UP library, varying from 68% to 99% similarity between them. Besides the much lower level of diversity among CTX-M-like genes from UP sites, the majority were similar to chromosomal ESBLs such as *bla*$_{RAHN-1}$. The results demonstrate that the occurrence and diversity of *bla*$_{CTX-M}$ genes are clearly different between polluted and unpolluted lotic ecosystems; these findings favor the hypothesis that natural environments are reservoirs of resistant bacteria and resistance genes, where anthropogenic-driven selective pressures may be contributing to the persistence and dissemination of genes usually relevant in clinical environments.

Antibiotics are widely used not only to treat human and animal infections but also in farms and aquacultures as food additives to promote animal growth and prevent diseases. Consequently, antibiotics are released in large amounts into natural ecosystems, where they can impact the structure and activity of environmental microbial populations (22, 23). Undoubtedly, the occurrence and dissemination of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are recognized worldwide as a major public health concern. Efforts for the prevention of the spread of ARGs and ARB focused on clinical and human community levels, being centered especially on infection control and the restriction of antibiotic use (34). However, considering growing evidence that ARGs and pathogenic ARB are no longer restricted to clinical settings, it is quite clear that research activities need to be expanded to include nonpathogenic environmental microorganisms that could be potential sources for these ARGs (22, 23, 36, 38).

Aquatic systems can be highly impacted by human activities, receiving contaminants and bacteria from different sources and thus encouraging the promiscuous exchange and mixture of genes and genetic platforms. Consequently, these systems may promote the spread of ARB and ARGs and even the emergence of novel resistance mechanisms and pathogens (2, 3, 38). Considering the frequent detection of ARGs and ARB in aquatic systems and that their dissemination constitutes a serious public health problem, it was suggested previously that ARGs should be considered emerging environmental contaminants (23, 29).

Beta-lactam antibiotics are the most broadly used antibacterial agents. Extended-spectrum beta-lactamases (ESBLs) mediate resistance to broad-spectrum beta-lactams such as cefotaxime (CTX) and ceftazidime and are widely disseminated among Gram-negative bacteria. Since first reported in 1983 (19), the occurrence of infections caused by ESBL-producing bacteria has been constantly rising and constitutes a serious threat to human health. CTX-M genes have rapidly become the most common ESBL genes, mainly because of the genetic platforms responsible for their mobilization and dissemination (insertion sequences, integrons, transposons, and plasmids). Particularly common in the genomic environment of these genes are insertion sequences such as IS*Ecp1*, IS*26*, and IS*CR1* (4, 5, 8). CTX-M-15 and CTX-M-14 are the most prevalent enzymes, with over 110 CTX-M-like ESBLs described so far, found mostly in the *Enterobacteriaceae* but also, for example, in *Aeromonas* spp., *Pseudomonas* spp., and *Acinetobacter* spp. (6, 8, 25, 35). Interestingly, the CTX-M-like ESBLs are thought to have evolved from chromosomal genes of the nonclinical genus *Kluyvera* (28). Few studies have addressed the links between pollution and the dispersal of ARB and ARGs in natural environments. It is of major importance to understand how anthropogenic activities are modulating the resistance gene pool in order to anticipate future impacts on and consequences for the environment and public health. Also, ARGs, and specifically those most frequently found in association with pathogenic bacteria, such as CTX-M genes, may be key indicators of water quality and may be used to trace the dissemination of multiresistance in aquatic environments.

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FIG 1 Map of the Vouga River basin (central Portugal) with the location of the 12 sampling sites under study (sites 1, 2, and 12 are polluted, and sites 3 to 9 are unpolluted).

In this study, our goal was to compare the cefotaxime resistomes within polluted (P) and unpolluted (UP) lotic (flowing water) ecosystems. Specific goals were (i) to compare the occurrence and phylogenetic diversity of cefotaxime-resistant (CTX^r) bacteria and ESBL producers, (ii) to detect and characterize the ESBL genes responsible for the resistance phenotype, and (iii) to compare the diversity of CTX-M-like genes using culture-dependent and culture-independent approaches.

MATERIALS AND METHODS

Sample collection and water quality assessment. Water samples were collected at 12 sites from 11 rivers integrated in the Vouga River basin, located in central Portugal (Fig. 1). Table S1 in the supplemental material indicates the global positioning system (GPS) coordinates of all sampling locations. Throughout the basin, these water bodies are exposed to different anthropogenic impacts from agricultural, industrial, and domestic origins, which results in different levels of superficial water quality from unpolluted to extremely polluted sites (11). Sampling sites were selected in order to include putative unpolluted to extremely polluted sites. Water was collected into sterile bottles (7 liters) from 50 cm below the water surface and kept on ice for transportation. To infer the water quality, physical, chemical, and microbiological parameters were determined according to Portuguese laws (10), which included pH, color, smell, dissolved oxygen, conductivity, temperature, nitrates, chlorides, phosphates, ammonium, chemical oxygen demand, biological oxygen demand, total and fecal coliforms, and fecal streptococci. Surface water quality classification was assigned according to regulations given by the National Institute of Water (www.inag.pt), which sorts water quality into 5 categories from unpolluted to extremely polluted water in accordance with parameters established by Portuguese law.

Enumeration and selection of cefotaxime-resistant bacteria. Water samples were filtered through 0.45-μm-pore-size cellulose ester filters (Pall Life Sciences, MI), and the membranes were placed onto MacConkey agar plates supplemented with 8 μg/ml of cefotaxime to select for cefotaxime-resistant isolates. Also, to determine the proportion of cefotaxime-resistant bacteria among the total bacterial population, plates with no antibiotic supplement were used. Plates were then incubated at 37°C for 16 h. Colony counting was done in triplicate. Individual cefotaxime-resistant colonies were purified and stored in 20% glycerol at −80°C.

Molecular typing and identification of cefotaxime-resistant isolates. Genomic DNA was isolated as previously described (17). BOX-PCR was used to type all isolates as previously described (32). PCR products were loaded onto 1.5% agarose gels for electrophoresis. The banding patterns were analyzed with GelCompar software (Applied Maths, Belgium). Similarity matrices were calculated with the Dice coefficient. Cluster analysis of similarity matrices was performed by the unweighted-pair group method using arithmetic averages (UPGMA). Isolates displaying different BOX profiles were identified by 16S rRNA gene sequencing analysis with primers and PCR conditions as previously described (17). PCR products were purified with the Jetquick PCR purification spin kit (Genomed, Löhne, Germany) and used as the template in the sequencing reactions. Online similarity searches were performed with the BLAST software at the National Center for Biotechnology Information website.

Antibiotic susceptibility testing and ESBL detection. Antimicrobial resistance patterns against the following 16 antibiotics from 6 classes were determined by the agar disc diffusion method on Mueller-Hinton agar: beta-lactams (penicillins; monobactams; carbapenems; and narrow-spectrum, extended-spectrum, and "fourth-generation" cephalosporins), quinolones, aminoglycosides, phenicols, tetracyclines, and the combination sulfamethoxazole-trimethoprim. Discs containing the following antibacterial agents were used: amoxicillin (10 μg), amoxicillin-clavulanic acid (20 μg/10 μg), ampicillin (10 μg), aztreonam (30 μg), cefepime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cephalothin (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), gentamicin (10 μg), imipenem (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), sulfamethoxazole-trimethoprim (25 μg), and tetracycline (30 μg) (Oxoid, Basingstoke, United Kingdom). After 24 h of incubation at 37°C, organisms were classified as sensitive, intermediate, or resistant according to Clinical and Laboratory Standards Institute guidelines (7). The detection of ESBL production was carried out by a double-disc synergy test (DDST) (18) and a clavulanic acid combination disc method, based on comparing the inhibition zones of cefpodoxime (10 μg) and cefpodoxime-plus-clavulanate (10 μg/1 μg) discs (Oxoid, United Kingdom). Statistical analysis was performed by a two-sample *t* test with a critical *P* value set at 0.05.

ESBL and integrase gene screening. PCR screening was performed for ESBL genes encoding SHV, TEM, OXA, CTX-M (groups 1, 2, 8/25, and 9), GES, VEB, and PER, with primer sets and PCR conditions described elsewhere previously (9, 16). Integrase screening was performed for the *intI1*, *intI2*, and *intI3* genes (9, 16, 24). Genomic DNA of positive-control strains was used (16, 24). Each experiment included a PCR mixture containing water instead of DNA as a negative control. Amplicons were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

Diversity and genetic environment of bla_{CTX-M} genes. Sequencing was done for the bla_{CTX-M} gene fragments amplified from the bacterial isolates. The presence of IS*Ecp1*, IS*26*, IS*5*, orf477, IS*903*, and orf503 in the genetic environment of bla_{CTX-M} was searched for by PCR (12, 13, 31).

Construction of bla_{CTX-M} gene libraries. To further investigate the diversity of the bla_{CTX-M} genes in both polluted and unpolluted environments, environmental DNA from water samples was isolated as previously described (17). DNAs isolated from all polluted sites were mixed, as was also done for unpolluted samples. Hence, two clone libraries of bla_{CTX-M} were constructed by using the TA cloning kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The bla_{CTX-M} gene was amplified by using primers CTX-F and CTX-R (21). Clones were screened by PCR for the presence of fragments with the expected size by using primers targeting the vector. PCR products were purified and sequenced. Similarity searches were performed by using BLAST. A phylogenetic tree was obtained using MEGA, version 5 (33). The Shannon-Weaver index of diversity (*H*) was calculated for each library by using the formula $H = -\Sigma(n_i/N)\log(n_i/N)$, where n_i is the abundance of each bla_{CTX-M}-like type and *N* is the sum of the analyzed clones in each library.

Nucleotide sequence accession numbers. All bla_{CTX-M} gene nucleotide sequences reported in this work have been deposited in the GenBank database under accession numbers JQ397652 to JQ397669 (bacterial strains) and JQ397670 to JQ397721 (clone libraries). Also, 16S rRNA gene sequences are available under accession numbers JQ781502 to JQ781652.

RESULTS

Water quality and occurrence of cefotaxime-resistant bacteria. From the analysis of all physical, chemical, and microbiological

parameters (see Table S2 in the supplemental material) and according to Portuguese law (10) and the surface water quality classification given by the National Institute of Water, of the 12 sites under study, 3 sites were classified as polluted (P) and 9 were classified as unpolluted (UP). All three rivers classified as polluted presented a mixed type of pollution, related mainly to exceptionally high levels of phosphates and total coliforms (see Table S2 in the supplemental material) (10).

The total bacterial count on MacConkey agar in polluted sites was on average 1.9×10^5 CFU/100 ml of riverine water, 8.8% of which grew on MacConkey agar supplemented with cefotaxime (1.7×10^4 CFU/100 ml), and that in pristine rivers was on average 0.68×10^5 CFU/100 ml, 0.6% of which grew on MacConkey agar supplemented with cefotaxime (4.4×10^2 CFU/100 ml).

Molecular typing and identification of bacterial isolates. Clonal relationships among cefotaxime-resistant isolates ($n = 225$) were assessed by BOX-PCR, and 151 isolates displaying unique BOX profiles were selected for further analysis (see Fig. S1 in the supplemental material). Among strains isolated from polluted waters ($n = 60$), 41.7% were identified as *Pseudomonas* spp. (*P. fluorescens*, *P. nitroreducens*, *P. plecoglossicida*, and *P. putida*), 35% were affiliated with members of the *Enterobacteriaceae*, and 21.7% were affiliated with *Aeromonas* spp. The members of the *Enterobacteriaceae* were affiliated mostly with *Escherichia coli* (25%), followed by *Enterobacter* spp. (8.33%) and only an isolate each of *Alcaligenes faecalis* and *Citrobacter freundii*.

Of the unpolluted water isolates ($n = 91$), 63.7% were *Pseudomonas* sp. isolates (*P. fluorescens*, *P. nitroreducens*, and *P. putida*); 8.8% and 1.1% were *Enterobacteriaceae* and *Aeromonas* sp. (*A. media* and *A. hydrophila*) isolates, respectively; and *Acinetobacter* sp. appeared to be second most abundant genus in these samples, with 26.4% of isolates (all *Acinetobacter calcoaceticus*). Among members of the *Enterobacteriaceae*, *Enterobacter* sp. and *Escherichia coli* isolates were identified (5.5% and 3.3%, respectively). A 16S rRNA gene phylogenetic tree is presented in Fig. S2 in the supplemental material.

Antimicrobial susceptibility and detection of ESBL producers. As expected, since isolates were selected in agar plates supplemented with cefotaxime, higher levels of antibiotic resistance were registered for beta-lactams (see Fig. S3 in the supplemental material). It was determined that 22.5% of the isolates from P and UP samples were resistant to all cephalosporins tested and that 52.3% were resistant to both cefotaxime and ceftazidime. For beta-lactams, higher percentages (although not statistically significant [$P > 0.05$ by a two-sample *t* test]) were always observed for isolates from polluted waters. For non-beta-lactam antibiotics, higher levels of resistance to quinolones (in particular, nalidixic acid, with 78.1% resistant isolates), sulfamethoxazole-trimethoprim, and chloramphenicol (55% and 51%, respectively) were observed. In isolates from polluted environments, resistance to tetracycline (36.7%) and to aminoglycosides (31.7%) was also frequently detected. Besides imipenem (99.3% susceptible strains), gentamicin was the most effective, with only 3.3% resistance among isolates from UP sites and 21.7% resistance among isolates from P sites. The less effective antimicrobial agents were the penicillins, the monobactam aztreonam, and narrow-spectrum and extended-spectrum cephalosporins. Significant differences in resistance frequencies among isolates from polluted and unpolluted waters toward aminoglycosides, quinolones, tetracycline, and the combination sulfamethoxazole-trimethoprim were found ($P < 0.05$ by a two-sample *t* test). Multiresistance (defined as resistance to 3 or more classes of antibiotics, including beta-lactams) was found in 56.6% and 46.0% of the strains isolated from polluted and unpolluted sites, respectively.

Of the 151 isolates tested, 39 were positive for ESBL production by both methods: 27 isolates from polluted waters (13 *Escherichia coli*, 8 *Aeromonas* spp., and 6 *Pseudomonas* spp.) and 12 isolates from unpolluted sites (7 *Pseudomonas* spp., 2 *Acinetobacter* sp., 2 *Escherichia coli*, and 1 *Aeromonas* spp.).

Occurrence and diversity of integrase and ESBL genes. The ESBL-producing isolates were further analyzed by PCR screening for ESBLs and integrase genes. For ESBL genes, the most frequently detected gene was $bla_{\text{CTX-M}}$ ($n = 18$), followed by bla_{TEM} ($n = 10$). For 6 strains, both $bla_{\text{CTX-M}}$ and bla_{TEM} were identified. Two bla_{VEB} genes were identified, both from *Aeromonas* sp. isolates, once in each environment. OXA-1-like genes were detected in 6 strains isolated from polluted sites. No bla_{GES}, bla_{PER}, bla_{SHV}, or $bla_{\text{OXA-2}}$- and $bla_{\text{OXA-10}}$-like genes were identified with the primer sets used in this study.

Among the 39 ESBL-producing isolates, the integrase genes *intI1*, *intI2*, and *intI3* were screened by PCR. In 22 out of the 39 isolates, the *intI1* gene was detected (19 P and 3 UP isolates), affiliated with *Escherichia coli* (11 P isolates and 1 UP isolate), *Pseudomonas* sp. (2 P isolates and 1 UP isolate), and *Aeromonas* sp. (6 P isolates and 1 UP isolate). The *intI2* and *intI3* genes were not detected.

Diversity and genetic environment of $bla_{\text{CTX-M}}$ genes. Since $bla_{\text{CTX-M}}$ was the gene most frequently detected among the 39 ESBL-producing isolates, $bla_{\text{CTX-M}}$ genes were further characterized (Table 1).

CTX-M genes were detected in 18 isolates (15 P and 3 UP isolates). The nucleotide sequences of the $bla_{\text{CTX-M}}$ genes were determined and their genomic environments were inspected by PCR and sequencing. Sequence analysis showed that isolates produced CTX-M from group 1 (CTX-M-1, -3, -15, and -32) and group 9 (CTX-M-14). The CTX-M-1 gene was found in 3 isolates (all from polluted water), the CTX-M-3 gene was found in 3 isolates (all from polluted water), the CTX-M-15 gene was found in 10 isolates (8 P and 2 UP isolates), and the CTX-M-32 gene was detected in only 1 isolate from unpolluted water. For group 9, the CTX-M-14 gene was found in one strain isolated from polluted water. The genetic environment study revealed the presence of 6 different genetic environments with elements previously described for clinical strains. A schematic representation of the different genomic environments found for the 18 isolates is presented in Fig. 2. IS*Ecp1* was found in the upstream region of all isolates examined in the present study but was disrupted in 8 isolates by IS*26* and in 1 isolate by IS*5*. The distance between IS*Ecp1* and the start codon of $bla_{\text{CTX-M}}$ genes was as previously described (12, 20, 31), varying from 32 bp to 127 bp. All $bla_{\text{CTX-M}}$ genes from group 1 presented Orf477 downstream. The only $bla_{\text{CTX-M}}$ gene from cluster 9 detected was $bla_{\text{CTX-M-14}}$ (isolate E6), which presented an IS*903*-like element downstream.

$bla_{\text{CTX-M}}$-like clone libraries from polluted and unpolluted environments. To compare the diversities of $bla_{\text{CTX-M}}$ genes in polluted and unpolluted environments, two clone libraries of $bla_{\text{CTX-M}}$-like gene fragments were constructed and analyzed. Gene fragments were amplified by using two environmental DNA pools, each corresponding to P and UP samples, as the template. A

TABLE 1 Characteristics of bla_{CTX-M}-producing isolates from polluted and unpolluted samples[a]

Isolate	Phylogenetic affiliation	Sample location	ESBL gene(s) detected by PCR	Antibiotic resistance profile[b]	*intI1* Presence
E1	*A. hydrophila*	P	bla_{TEM}, bla_{CTX-M}	AML, AMP, AMC, KF, CTX, FEP, CIP, NA, CN, K, TE	+
E2	*A. hydrophila*	P	bla_{TEM}, bla_{CTX-M}	AML, AMP, AMC, KF, CTX, FEP, NA, CN, K, TE	+
E3	*A. hydrophila*	P	bla_{TEM}, bla_{CTX-M}	AML, AMP, AMC, ATM, KF, CTX, FEP, CIP, NA, K, TE	+
E4	*E. coli*	P	bla_{CTX-M}	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, CIP, NA, C, CN, K, TE	−
E5	*E. coli*	P	bla_{CTX-M}, bla_{OXA}	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, CIP, NA, CN, K, SXT, TE	+
E6	*E. coli*	P	bla_{TEM}, bla_{CTX-M}	AML, AMP, AMC, ATM, KF, CTX, NA, C, TE	+
E7	*E. coli*	P	bla_{TEM}, bla_{CTX-M}, bla_{OXA}	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, CIP, NA, CN, K, SXT, TE	+
E8	*E. coli*	P	bla_{CTX-M}, bla_{OXA}	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, CIP, NA, CN, K, SXT, TE	+
E9	*E. coli*	P	bla_{CTX-M}	AML, AMP, ATM, KF, CTX, CAZ, FEP, CIP, NA, CN, K, SXT, TE	+
E10	*E. coli*	P	bla_{CTX-M}	AML, AMP, ATM, KF, CTX, CAZ, FEP, CIP, NA, K, SXT, TE	+
E11	*E. coli*	P	bla_{CTX-M}	AML, AMP, ATM, KF, CTX, CAZ, FEP, CIP, NA, K, SXT, TE	+
E12	*E. coli*	P	bla_{CTX-M}, bla_{OXA}	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, CIP, NA, CN, K, SXT, TE	+
E13	*E. coli*	P	bla_{TEM}, bla_{CTX-M}	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, SXT	+
E14	*E. coli*	P	bla_{CTX-M}	AML, AMP, ATM, KF, CTX, FEP, SXT, TE	+
E15	*E. coli*	P	bla_{CTX-M}	AML, AMP, AMC, ATM, KF, CTX, CAZ, FEP, NA, SXT, TE	+
E16	*E. coli*	UP	bla_{CTX-M}	AML, AMP, ATM, KF, CTX, CAZ, FEP, CIP, NA, SXT	+
E17	*E. coli*	UP	bla_{TEM}, bla_{CTX-M}	AML, AMP, AMC, ATM, KF, CTX, CAZ, TE	−
E18	*Pseudomonas* sp.	UP	bla_{CTX-M}	AML, AMP, AMC, ATM, KF, CTX, NA, C, SXT	+

[a] Data shown include phylogenetic affiliations, sample origins, ESBL and integrase genes detected, and antimicrobial resistance profiles.
[b] AML, amoxicillin; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; KF, cephalotin; FEP, cefepime; CIP, ciprofloxacin; NA, nalidixic acid; CN, gentamicin; K, kanamycin; TE, tetracycline; ATM, aztreonam; CAZ, ceftazidime; SXT, trimethoprim-sulfamethoxazole; C, chloramphenicol.

total of 52 clones were obtained, and all inserts were sequenced (27 P and 25 UP isolates). Culture-independent bla_{CTX-M}-like gene libraries comprised 16 gene variants (variants A to P): 14 types in the P library ($H = 1.04$) and 4 types in UP library ($H = 0.23$), with similarity values ranging from 68% to 99% between them and from 97% to 100% with sequences from the GenBank database. The majority ($n = 16$) were affiliated with nucleotide sequences of bla_{CTX-M} variants from group 1 (CTX-M-1, -12, -15, -30, -37, -68, and -97), but bla_{CTX-M} variants from group 2 (CTX-M-97) ($n = 2$), group 9 (CTX-M-14) ($n = 3$), and group 25 (CTX-M-78 and -100) ($n = 2$) were also identified.

Besides the much lower level of diversity among UP CTX-M-like genes, the majority were similar to chromosomal ESBLs such as bla_{RAHN-1}, bla_{RAHN-2}, and bla_{FONA-5} (Fig. 3).

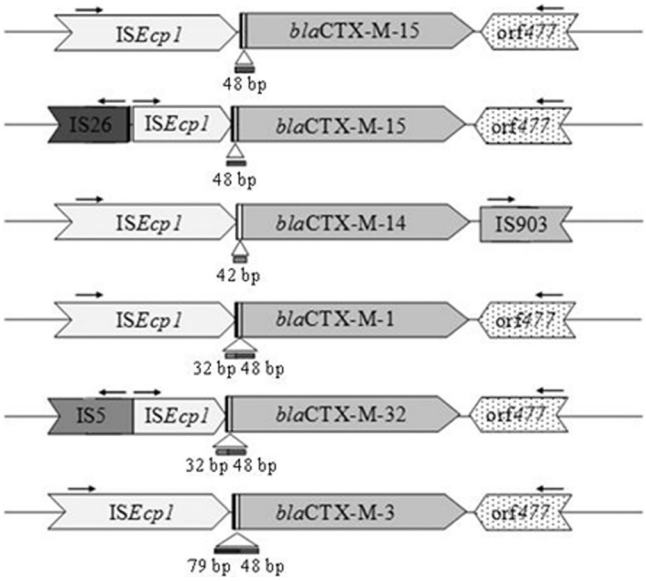

FIG 2 Schematic representation of the genetic environment of CTX-M genes from the 18 isolates producing CTX-M from group 1 (CTX-M-1, -3, -15, and -32) and group 9 (CTX-M-14). The numbers of isolates from each polluted and unpolluted environment that carry each variant are indicated.

DISCUSSION

Lotic ecosystems are threatened daily by anthropogenic actions that compromise the quality of water and, as a consequence, its sustainable use.

Considering aquatic systems as reactors for diverse biological interactions that have important genetic implications, the study of the aquatic antibiotic resistome (which includes ARGs and pathogenic and nonpathogenic ARBs) is important, as it may indicate the extent of the alteration of water ecosystems by anthropogenic activities. Several previous studies reported the presence of antibiotic-resistant bacteria from several aquatic environments but focused on pathogenic organisms or those directly related to an environmental threat, such as hospital sewage discharges (1, 3, 36).

In this study, two groups of rivers (polluted and unpolluted), which are part of the same Portuguese lotic ecosystem, were inspected for the presence of cefotaxime-resistant Gram-negative bacteria, in order to understand how human action is modulating the environmental resistome, in particular the cefotaxime resistome.

As expected, high levels of resistance against other beta-lactams among CTX^r isolates, frequently conferred by the same resistance mechanism, were found in this study (16), with a higher level of occurrence among strains from P environments. ESBL production was detected in *Pseudomonas* sp., *Acinetobacter* sp., *Escherichia coli*, and *Aeromonas* sp. isolates and was found more frequently among isolates from polluted sites. Recently, in several environmental studies, members of the same genera have been identified as ESBL producers, enforcing their relevance and importance for resistance monitoring (14, 15, 27). We investigated the presence of different ESBL genes and found bla_{CTX-M} genes to be the most prevalent, followed by bla_{TEM} genes. The majority of the isolated CTX-M-producing strains were affiliated with *E. coli* but also with *Aeromonas hydrophila* (3 isolates producing $bla_{CTX-M-3}$) and *Pseudomonas* sp. (1 isolate producing $bla_{CTX-M-15}$).

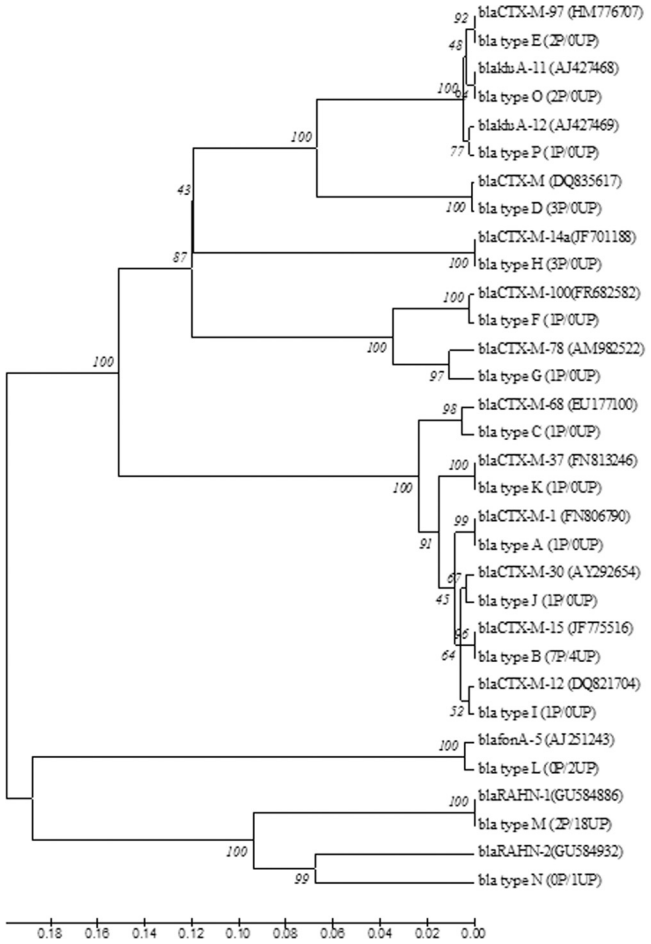

FIG 3 Dendrogram tree of bla_{CTX-M} gene sequence types A to P identified from the polluted (P) and unpolluted (UP) genomic libraries. The numbers in parentheses show the number of times that the sequence was found in the library. The branch numbers refer to the percent confidences, as estimated by a bootstrap analysis with 1,000 replications.

Few studies have reported the presence of bla_{CTX-M} genes in *Pseudomonas* spp. and *Aeromonas* spp. A previous study reported the presence of $bla_{CTX-M-27}$ genes in 2 *Aeromonas* sp. strains isolated in river sediment (21). Also, *Aeromonas* sp. isolates producing $bla_{CTX-M-3}$ and $bla_{CTX-M-15}$ were detected previously in clinical settings and were directly implicated in human infections (37). As far as we know, this is the first work to report environmental *Aeromonas* sp. isolates producing $bla_{CTX-M-3}$ genes. Also, for *Pseudomonas* spp., reports of CTX-M-producing isolates have been rare. In fact, the majority of those studies referred to clinical *Pseudomonas aeruginosa* isolates, which were reported to produce CTX-M-1, -2, -15, and 43 (26). Also, 2 spinach saprophyte strains recently identified as *P. putida* and 1 strain identified as *Pseudomonas teessidea* were referred to as CTX-M-15-producing strains (30).

To detect any potential genetic platforms able to mobilize the bla_{CTX-M} genes, we also analyzed the genomic environment of the 18 bla_{CTX-M} genes detected. Different insertion sequence elements were found. An IS*Ecp1* element upstream of the *bla* gene was detected in all strains. Other IS elements (IS*5* and IS*26*) were found but were disrupting the IS*Ecp1* element. The organization of IS*26* and the end of IS*Ecp1* has been found mostly in clinical *Enterobacteriaceae* isolates but was also described for an *E. coli* $bla_{CTX-M-1}$-producing strain isolated from fecal droppings of seagulls (12, 27, 31). On the other hand, the organization of IS*5* and the end of IS*Ecp1* was found upstream of the $bla_{CTX-M-32}$ gene in environmental and clinical *E. coli* isolates (13, 27). The presence of an IS*Ecp1* element upstream of $bla_{CTX-M-1}$, $bla_{CTX-M-3}$, $bla_{CTX-M-14}$, and $bla_{CTX-M-15}$ in clinical isolates was also reported previously (12, 20, 31). Downstream of the *bla* genes in the CTX-M-1 group, the Orf477 sequence was present in all strains. Another insertion sequence, IS*903*, was found downstream of the $bla_{CTX-M-14}$ gene from CTX-M group 9, as described previously for clinical *Enterobacteriaceae* isolates (12, 20, 31). The common phenotype of multiresistance among ESBL-producing isolates is a result of the presence of other genes normally carried on the same plasmid carrying ESBL genes. This gene panoply contributes to the maintenance of ESBL-producing bacterial communities, even with a low concentration of beta-lactams (8). As reported in this work, it is of particular concern that 88.9% of the CTX-M-producing isolates are multiresistant (93.3% P and 66.6% UP isolates). Among CTX-M producers isolated from polluted waters, resistance to quinolones, aminoglycosides, tetracyclines, and the combination sulfamethoxazole-trimethoprim was highly prevalent. Due to their ability to capture and incorporate gene cassettes from the environment, integrons have an important role in the spread of multidrug resistance in Gram-negative bacteria. In this work, class 1 integrons were detected in 56.4% of ESBL-producing isolates (48.7% in P and 7.7% in UP sites).

Analysis of only the cultivable fraction of Gram-negative bacteria in MacConkey agar plates might underestimate the diversity of bla_{CTX-M} gene variants present in the lotic ecosystem under study. To overcome this methodological aspect, a culture-independent approach was applied to further analyze the diversity of bla_{CTX-M} genes in both environments. For this, two clone libraries of bla_{CTX-M} gene fragments amplified from polluted and unpolluted environmental DNA were constructed and analyzed. In the P library, the variety of CTX-M-like genes was much greater than that in the UP library. This is probably related to the greater anthropogenic selective pressures posed by the release of antibiotics and/or antibiotic-resistant bacteria. Also, other studies showed previously that other contaminants can also contribute to the persistence of antibiotic resistance in the environment, like, for example, heavy metals and disinfectants (22, 23). Within the P library, similarities with bla_{CTX-M} genes from 4 clusters and also with chromosomal variants referred to as ancestors of clusters CTX-M-1 and CTX-M-2 were found. Interestingly, the majority of the bla_{CTX-M}-like sequences found in unpolluted DNA were similar to those of chromosomal class A ESBLs that were described previously for *Rahnella* spp. (bla_{RAHN-1} and bla_{RAHN-2}) and *Serratia fonticola* (bla_{FONA-5}). In a previous study, a bla_{CTX-M} library cloned from urban river sediment DNA also presented a high level of diversity of bla_{CTX-M} sequences, with 13 variants being found (21). Overall, the results presented here show clear differences in polluted and unpolluted environments. While we found at most 4 variants in unpolluted rivers, with the majority being related to ancestor chromosomally located genes, up to 14 variants were found in polluted waters (from 4 out of 5 clusters identified so far for CTX-M enzymes).

A shift in the distribution of different ESBLs has recently occurred in European clinical settings, with a dramatic increase in

CTX-M enzymes over TEM and SHV variants. More than 110 CTX-M variants have been described so far. Due to the high level of homology with chromosomal beta-lactamases from different *Kluyvera* species, these variants are now recognized as CTX-M ancestors, such as KLUA-1 from *K. ascorbata* and KLUG-1 from *K. georgiana* (5). However, the diversity that we found at polluted sites cannot be attributed to the presence of bacteria carrying ancestral CTX-M genes. As for clinics, our results suggest that CTX-M gene dominance is correlated to selective pressures imposed by human activities.

These findings sustain our hypothesis that anthropogenic activities might modulate the environmental resistance gene pool and promote antibiotic resistance dissemination. Also, we have shown that ESBL genes are a form of environmental pollution resulting either from the intake of ARGs or ARB from human activities or from the selection of resistant environmental bacteria by subtherapeutic antibiotic doses released into the environment. In our study, ESBL genes were found in genera not included in routine evaluations of water quality, associated with the genetic platforms needed for their mobilization and transfer. Thus, we suggest that data on the occurrence and diversity of ESBL genes, and specifically CTX-M genes, can be used to assess ecosystem health and antibiotic resistance evolution. However, more studies of other geographical locations are needed to validate this application. These genes are also good candidates to be used as pollution indicators. To further confirm this potential, source-tracking approaches must be conducted to link the presence of CTX-M genes to specific sources of contamination.

Conclusions. The work here presented showed that the occurrence and antimicrobial susceptibility profiles of CTX^r bacteria are markedly different between polluted and unpolluted lotic ecosystems; the same happens with the occurrence and diversity of clinically relevant ESBL genes. Our results validate the hypothesis that anthropogenic impacts on water environments are modulators of the resistance gene pool and promote the dissemination of antibiotic resistance.

In addition, this work suggests that bla_{CTX-M}-like genes may constitute indicators of pollution by antibiotics, which is useful for the study of the dispersal of antibiotic resistance in aquatic environments.

We also conclude that the dissemination of resistance to broad-range antibiotics such as cefotaxime may be at an earlier stage in pristine environments, providing the opportunity for continuing studies of the impact of anthropogenic-driven dissemination and evolution.

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